Enhancing the possibilities of LCMS/MS for the absolute quantification of proteins in biological samples

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Absolute quantification of the total and anti-drug antibody-bound concentrations of recombinant human α-glucosidase in human plasma using protein-G extraction and LC-MS/MS.

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5.1 Abstract.

The administration of protein-based pharmaceuticals can cause the in vivo formation of anti-drug antibodies (ADAs) which may reduce the efficacy of the therapy by binding to the protein drug. An accurate determination of the total and ADA-bound concentrations of the drug gives information on the extent of this immune response and its consequences and may help develop improved therapeutic regimens. We present an absolute quantitative method to differentiate between total, free and ADA-bound drug for recombinant human alpha acid glucosidase (rhGAA) in plasma from patients suffering from Pompe's disease.

LC-MS/MS quantification of a signature peptide after trypsin digestion of plasma samples before and after an extraction of the total IgG content of plasma with Protein-G coated beads was used to determine the total and the ADA-bound fractions of rhGAA in samples from Pompe patients after enzyme infusion. The methods for total and ADA-bound rhGAA allow quantification of the drug in the range of 0.5 to 500 µg/mL using 20 µL of plasma and met the regular bioanalytical validation requirements, both in the absence and presence of high levels of anti-rhGAA antibodies. This demonstrates that the ADA-bound rhGAA fraction can be accurately and precisely determined and is not influenced by sample dilution, repeated freezing and thawing or extended bench-top or frozen storage.

In samples from a patient with a reduced response to therapy due to ADAs high ADA-bound concentrations of rhGAA were found, while in the samples from a patient lacking ADAs, no significant ADA-bound concentrations were found.

Since Protein G captures the complete IgG content of plasma, including all anti-drug antibodies, the described extraction approach is universally applicable for the quantification of ADA-bound concentrations of all non-IgG-based biopharmaceuticals.

5.2 Introduction.

Reliable analytical methods for the quantitative determination of biopharmaceutical proteins in biological samples are indispensable for drug development as well as therapeutic drug monitoring once on the market.
Traditionally, large biopharmaceutical proteins are quantified with ligand binding assays (LBAs), but in recent years liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has been gaining popularity in this area[1,2], usually in combination with proteolytic digestion of the protein analyte into a series of peptides, one or more of which are quantified as a surrogate for the intact protein. One reason for the increased interest for LC-MS/MS is its generally better quantitative performance, caused by the absolute nature of the measurement and the possibility to correct for experimental variability by using stable-isotope labeled internal standards[3,4]. Furthermore, LBAs can be influenced by interactions between the analyte and other proteins in the sample[5,6], whereas the proteolytic digestion typically used in LC-MS/MS workflows circumvents this problem since it cleaves all proteins in the sample and thereby also disrupts interactions with other proteins.
One of the main classes of proteins that interfere in LBAs are anti-drug antibodies (ADAs)[7,8], which are elicited by the patient's immune system in an attempt to clear foreign and unknown substances, in this case the drug, from the body. In LBAs, ADAs can compete with both the capture and detection antibodies for binding sites on the drug. Therefore, the presence of ADAs in a sample typically results in an underestimation of the total amount of drug, when quantified using an LBA[7,9]. In contrast, LC-MS/MS allows an unbiased determination of the total drug concentration in ADA-positive samples.
Next to the total concentration, it may be valuable to also determine the ADA-bound concentration of a drug, e.g. to correlate pharmacokinetic data to the pharmacological activity of the protein. To this end, free and ADA-bound drug have to be separated, after which digestion and LC-MS/MS analysis can be applied to the isolated fractions.
One possibility to extract an ADA-bound biopharmaceutical from a sample is based on the interaction of the constant region of immunoglobulins with protein A, G or L. These proteins have different affinities towards the immunoglobulin subclasses. IgM, the first immunoglobulin to be released upon exposure to an antigen, is only significantly bound by protein L, which makes this the preferred reagent for isolation of ADA-bound drug in samples from clinical trials with previously drug-naïve healthy volunteers. In therapeutic drug monitoring of patients who are on prolonged therapy, the ADA-population will mainly consist of IgG subclasses to which both protein A or G display a high affinity.
Protein G-based extraction has been combined with LC-MS/MS to assess the total ADA binding capacity in plasma by the addition of excess drug, isolation of the ADA-

drug complex and quantification of a signature peptide of the protein drug as a measure for the presence of total ADA[10]. In addition, the combination of protein A/G-based enrichment with LC/MS-MS has been investigated in an exploratory method for the ADA-bound fraction of a 4.5kDa therapeutic peptide in a comparison with ultrafiltration on a 100kDa molecular weight cutoff filter to separate free from ADA-bound peptide[11]. In our work, we describe the combination of LC-MS/MS and protein-G for the absolute quantitative determination of the total as well as the ADA-bound concentrations of a large biopharmaceutical protein and we describe several critical experiments required for the successful development of such a method.

The method was developed for the 110-kDa biopharmaceutical drug recombinant human alpha acid glucosidase (rhGAA), which is used to treat patients suffering from glycogen storage deficiency type II, or Pompe's disease, in which genetic defects prevent the expression of a (fully functional) acid alpha-glucosidase protein[12]. Absence of the enzyme results in storage of glycogen in several tissues, especially skeletal muscle and heart. This results in progressive muscle weakness, cardiac failure and reduced life expectancy of patients. Enzyme replacement therapy (ERT) with rhGAA has shown to reduce glycogen storage and improve life expectancy of patients[13–15]. Determination of the ADA-bound rhGAA fraction is relevant, as a large part of the patient population develops an immune response to the drug, which reduces the efficacy of ERT.

In normal clinical practice, the circulating amount of rhGAA in dosed patients is quantified by measuring its activity[13], a technique which is often complemented with a protein-A depletion to determine the free (non-ADA bound) activity. This approach generates results for the free and total activity in plasma, from which the ADA-bound fraction can be calculated. However it does not give insight in the actual concentrations of total and ADA-bound rhGAA as the data might be biased by to the presence of neutralizing ADAs which render the protein drug inactive and thus impact the measured total activity of the drug. This potentially leads to a misrepresentation of the extent of ADA-binding which can complicate the correct adjustment of the dosage regime to restore therapeutic efficacy. The use of LC-MS/MS with a protein-G extraction described in this paper allows the correct determination of the total and ADA-bound rhGAA concentrations, regardless of the presence of neutralizing antibodies. To confirm the suitability of the approach we report the results of a validation of the methods for total and ADA-bound rhGAA and its application to plasma samples taken from different patients suffering from Pompe's disease.

5.3 Materials and Methods.

5.3.1 Chemicals.

Methanol, acetonitrile, formic acid, Tween-20, ammonium bicarbonate, Tris(hydroxymethyl)aminomethane (Tris), non-N-Tosyl-L-phenylalanine chloromethyl ketone treated trypsin from porcine pancreas, ammonium acetate, disodium hydrogenphosphate, sodium dihydrogenphosphate, hydrochloric acid, glycine and sodium chloride were obtained from Sigma Aldrich (St. Louis, MO, USA). HPLC grade water was prepared using a water purification system from Merck-Millipore (Darmstadt, Germany). The stable-isotope labeled (SIL) internal standard, a peptide with amino acid sequence VTSEGAGLQLQK, in which the C-terminal lysine contained a $^{13}C_6{}^{15}N_2$ isotope label, was obtained from JPT-peptides (Berlin, Germany).

5.3.2 Biological materials.

Recombinant human alpha acid glucosidase (rhGAA) (Myozyme®) was obtained from Genzyme corporation (Cambridge, MA, USA). Drug- and ADA-free human plasma on K2-EDTA from healthy volunteers (hereafter referred to as blank human plasma) was obtained from Seralabs (Haywards Heath, UK) Drug-free and ADA-containing human plasma was obtained by pooling individual aliquots of (K2-EDTA) plasma collected from Pompe patients that had been on rhGAA for varying periods of time and had developed relatively high ADA titers, prior to drug administration and after complete washout of the previous dose.

5.3.3 Preparation of samples.

A rhGAA stock solution of 5.0 mg/mL was prepared by dissolving the contents of a vial of freeze-dried protein (label claim: 50 mg) in 10.3 mL of water according to the manufacturer's instructions for use. The stock solution was divided into 0.400mL aliquots in polypropylene tubes and stored at -80 ºC. From this stock solution calibration samples were prepared in blank human plasma at 0.500, 1.00, 2.50, 10.0, 25.0, 100, 250, 400 and 500 µg/mL. Similarly, validation and quality control (QC) samples were prepared at 0.500, 1.50, 40.0 and 400 µg/mL. In addition, two positive control (PC) samples were prepared by spiking drug-free, ADA-containing plasma with rhGAA. For PC-A (high drug, low ADA), rhGAA was spiked at 125 µg/mL in patient plasma that had been diluted ten-fold with blank human plasma. Likewise, PC-B (low drug, high ADA) was prepared at 9.23 µg/mL rhGAA patient plasma 2.5-fold diluted with blank human plasma. All calibration, validation, QC and PC samples were stored in polypropylene tubes at -80 ºC.

5.3.4 Sample pretreatment.

For the determination of the total rhGAA concentration in both ADA-free and ADA-containing plasma, 20-µL aliquots of sample were pipetted into the 1-mL wells of a 96-well plate and 400 µL of methanol was added. After vortex-mixing for 1 minute, the proteins were allowed to precipitate at room temperature for 10 minutes after which they were pelleted by centrifugation for 10 minutes at 1000g. The supernatant was discarded by inverting the plate above a waste receptacle and placing it upside down on a clean paper towel for 5 minutes. The protein pellet was reconstituted by vortex-mixing in 200 µL of a solution consisting of 50 mM ammonium bicarbonate at pH8.2, 0.05% Tween-20 and 200 µg/mL trypsin and which contained 75 ng/mL of the SIL-peptide internal standard. The sample was digested at 37°C and 900 rpm for 3 hours, after which the digestion was stopped by the addition of 50 µL of a solution of 5% formic acid in water. Finally, the plate was sealed, vortex-mixed and placed in the autosampler at 10°C for analysis.
For the determination of the ADA-bound rhGAA concentration, 0.2-mL Nab^{tm} Protein-G spin columns (Thermo Scientific, Waltham, MA, USA) were used, from which the storage solvent was removed before use by centrifuging for 2 minutes at 2000g. The beads were then reconstituted in 380 µL of load/wash solution (100 mM phosphate buffer at pH 7.2, 100 mM sodium chloride and 0.05% Tween-20), after which 20 µL of plasma was added. The complex of Protein-G and ADA-bound rhGAA was allowed to form for 30 minutes at room temperature on a rotation homogenizer, after which the supernatant containing unbound rhGAA was removed by centrifugation for 2 minutes at 2000g and discarded. The beads were reconstituted in 400 µL of load/wash solution and washed by overhead mixing on a rotation homogenizer for ten minutes, after which the washing solvent was removed by centrifugation for 2 minutes at 2000g and discarded. ADA-bound rhGAA was released from the protein-G beads during 10 minutes on a rotation homogenizer after the addition of 400 µL of 200 mM Glycine-HCl buffer at pH 2.0, which contained 100 mM sodium chloride and 0.05% Tween-20. After centrifugation for 2 minutes at 2000g, the supernatant was collected in a 2-mL sealable tube, to which 1600 µL of methanol, and 140 µL of 500 mM solution of Tris (pH 11) were added. After vortex- mixing for 1 minute, the proteins were allowed to precipitate at room temperature for 10 minutes after which they were pelleted by centrifuging for 10 minutes at 10000g. The supernatant was discarded and the internal standardization and digestion of the pellets was performed in an identical manner as for the total rhGAA method. To prevent carry over, the protein-G columns were washed after each use with 400 µL of 200 mM Glycine-HCl buffer at pH 2.0, which contained 100 mM sodium chloride and 0.05% Tween-20. After 10 minutes of washing, the solvent was removed by centrifugation for 2 minutes at 2000g. The beads were

reconstituted and stored before re-use in 100 mM phosphate buffer at pH 7.2, 100 mM sodium chloride and 0.05% Tween-20.

5.3.5 Instrumental analysis.

The chromatographic separation was performed using an I-class UPLC system (Waters, Milford, MA, USA) equipped with a 150x2.1mm Acquity PST CSH130 C18 column with 1.7-µm particles (Waters). The mobile phases where 0.1% formic acid in water (A) and acetonitrile (B). Gradient elution was performed at a flow-rate of 0.4 mL/min by a linear increase of mobile phase B from 6% to 13.5% in 15 minutes followed by a 2-min washing step at 90%B to clean the column. For detection, a Xevo-TQS triple quadrupole mass spectrometer (Waters) was used with an ionization voltage of 3500 V and a gas temperature of 400 °C. Other settings as well as the monitored SRM transitions for the signature peptide and internal standard are shown in Table 1.

Peptide	sequence	ion	fragmen	Cone	Collision
	(m/z)	(m/z)	(V)	(V)	
rhGAA C-side	VTSEGAGLQLQK	$[m+2]^{2+}$ / 615.8	$y10^{1+}$ / 1030.6	30	18
Int. Std.	VTSEGAGLQLQK[a]	$[m+2]^{2+}$ / 619.8	$y10^{1+}$ / 1038.6	30	18
Human IgG	ALPAPIEK	$[m+2]^{2+}$ / 419.8	$y5^{1+}$ / 557.3	40	20
HSA	LVNEVTEFAK	$[m+2]^{2+}$ / 575.3	$y8^{1+}$ / 937.5	40	30
rhGAA N-side	YEVPLETPR	$[m+2]^{2+}$ / 552.3	$y6^{1+}$ / 712.4	40	20
rhGAA N-side	TTPTFFPK	$[m+2]^{2+}$ / 469.8	$y6^{1+}$ / 736.4	40	20

[a] $^{13}C_6{}^{15}N_2$ labeled.

Table 1. *SRM transitions and mass spectrometric settings*

5.3.6 Method validation.

Total rhGAA concentrations in the validation and positive-control samples were determined by applying the method for total rhGAA and comparing the results to a calibration curve spiked with rhGAA in blank, ADA-free plasma, which was analyzed with the same method in the same run. The method for total rhGAA determination was validated according to international guidelines for chromatographic methods[16–18] by assessing precision, accuracy, selectivity and stability using calibrators and validation samples in ADA-free plasma. Besides the regulatory validation requirements, an additional selectivity assessment was performed to determine the performance of the total rhGAA method in ADA-containing positive control plasma samples.
Quantification of ADA-bound rhGAA in the positive-control samples was performed by application of the method for bound rhGAA and comparing the results to a calibration curve spiked with rhGAA in ADA-free plasma, which was simultaneously analyzed using the method for total rhGAA. The ability of the method to correctly quantify rhGAA in the eluate of the protein-G material (which has a much reduced protein content) against a calibration curve in unextracted plasma was assessed. To this end, blank plasma was extracted using the protein G cartridges to create blank eluate, which was subsequently spiked with rhGAA at two concentrations (1.50 and 400 µg/mL), processed according to the presented method and analyzed against a calibration curve in plasma that was directly digested.The method for ADA-bound rhGAA was validated by evaluating precision, accuracy, and stability. A detailed description of all validation experiments is included in the supplemental data.

5.3.7 Clinical background of the patients.

The patients in this study were receiving intravenous doses of 20 mg/kg rhGAA once per two weeks. A patient with a normal response to enzyme replacement therapy (ERT) and an ADA titer of 50, determined with Elisa, was dosed according to the regular regimen[19] which consisted of an initial 5 mL/h for 30 minutes, followed by 20 mL/h for the next 30 minutes, then 88 mL/h for another 30 minutes and 250 mL/h until the end of infusion. Due to infusion related reactions, another patient with a reduced response to ERT and an ADA titer of 3906250, also determined with Elisa, was dosed according to a slightly altered regimen, which consisted of an initial 5 mL/h for 60 minutes, followed by 20 mL/h for the next 60 minutes, then 88 mL/h for another 60 minutes and 180 mL/h until the end of infusion, while the total dose remained the same. Blood samples were drawn and plasma was prepared at set time-points during and after dosing. The patients described in this report had given informed consent for all studies they participated in.

5.4 Results and Discussions.

5.4.1 Signature peptide selection.

The protein rhGAA contains 952 amino acids, has six glycosylation sites and a molecular mass of approximately 110 kDa. An *in silico* trypsin digestion, performed with mMass[20], revealed the theoretical formation of 61 different peptides, 16 of which were deemed useable for quantification based on their size, the absence of glycosylation and unstable (methionine) and disulfide forming (cysteine) amino acids in their sequence. Eight of these were identified by LC-MS in a trypsin digest of the rhGAA stock solution. Comparison of their sequences to the non-redundant sequences of the known human proteome, using the basic local alignment search tool (BLAST) version 2.2.29[21], showed that they were all unique for human alpha acid glucosidase.

The three peptides with the highest sensitivity and selectivity in single reaction monitoring (SRM) mode were used for further method development: one from the C-terminal region and two from the N-terminal region of rhGAA. The peptide from the C-terminal region (VTSEGAGLQLQK) demonstrated the best performance, and was selected to be used for the quantification of rhGAA, and a SIL analogue of this peptide was obtained for use as an internal standard. The other two peptides (YEVPLETPR and TTPTFFPK) were used for qualitative purposes to confirm the presence of rhGAA. To monitor the selectivity of the protein-G extraction towards IgG, two other peptides were also monitored in each sample: ALPAPIEK, from the constant region of the heavy chain as signature peptide for human IgG1[22] and LVNEVTEFAK as signature peptide for human serum albumin (HSA)[23]. The SRM transitions of all monitored peptides are shown in Table 1.

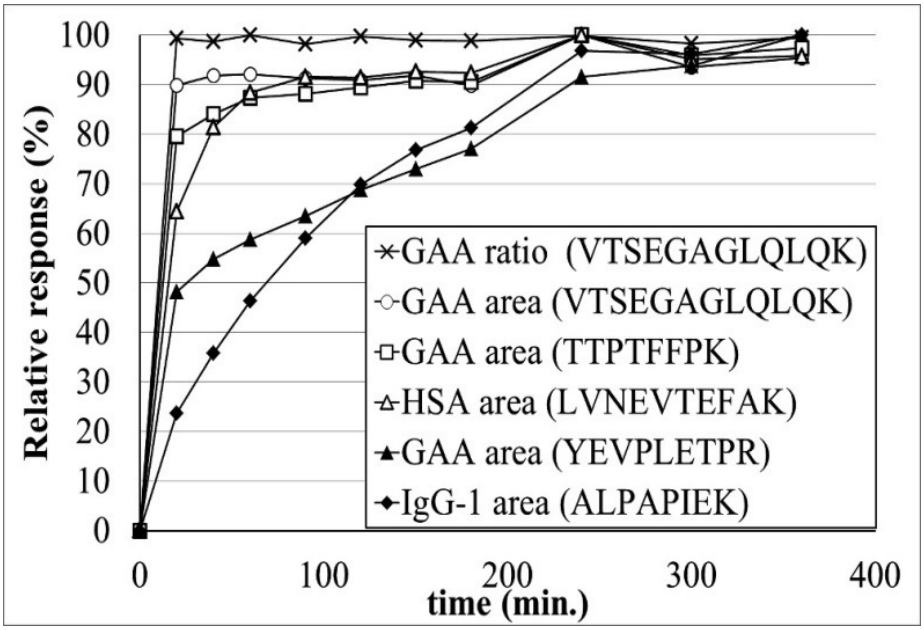

Figure 1. *Digestion-time course for all monitored peptides. showing the peak areas (for VTSEGAGLQLQK also ratio over internal standard) expressed as a percentage of the highest measured value for each peptide.*

5.4.2 Trypsin digestion.

The time-course for the release of all monitored tryptic peptides during the digestion step is shown in Figure 1. Considerable differences in the speed of release between the peptides were observed. For the rhGAA peptide VTSEGAGLQLQK, the results indicate that the peptide was already completely released at the first time-point of 20 minutes of digestion. In contrast, another rhGAA peptide (YEVPLETPR) needed over 6 hours to reach the plateau indicating its complete release. This difference may at least in part be attributed to the fact that for the peptide YEVPLETPR, the N-terminal amino acid (Y) is connected to an RR sequence in the intact protein. These so called ragged ends are known for high rates of missed cleavages and poor digestion reproducibility, even after long digestion periods[24]. Furthermore, differences in the accessibility of the cleavage sites for trypsin might also cause this effect. Comparison of the results for VTSEGAGLQLQK obtained using peak area with those using area ratio over internal standard shows the normalizing effect of the internal standard.

For the final method, we selected a digestion period of three hours even though a much shorter period would have sufficed to fully release the quantifying signature

peptide for rhGAA. This was done to allow a reasonable release (>80%) of the third peptide for rhGAA and for the IgG-1 specific peptide ALPAPIEK, as well as to prevent detrimental effects from the possible injection of partly digested proteins, which might cause fouling of the LC-MS/MS system.

5.4.3 Chromatographic separation and detection.

The chromatographic method proved sufficiently selective to separate all monitored peptides from the observed peaks in the LC-MS/MS chromatogram which result from the myriad of matrix peptides released by trypsin from the plasma proteome. To achieve this, a relatively shallow gradient of 0.5% per minute increase in modifier content in the mobile phase was combined with the 150-mm analytical column with 1.7µm particle diameter.

In Figure 2, all monitored SRM transitions are shown for a sample with an rhGAA concentration of 2.50 µg/mL; IgG and HSA were at endogenous plasma levels. The presented method was used to analyze almost 1000 injections using one analytical column, which did not show decreased performance or increased backpressure which indicates that (partly) undigested proteins or did not cause problems.

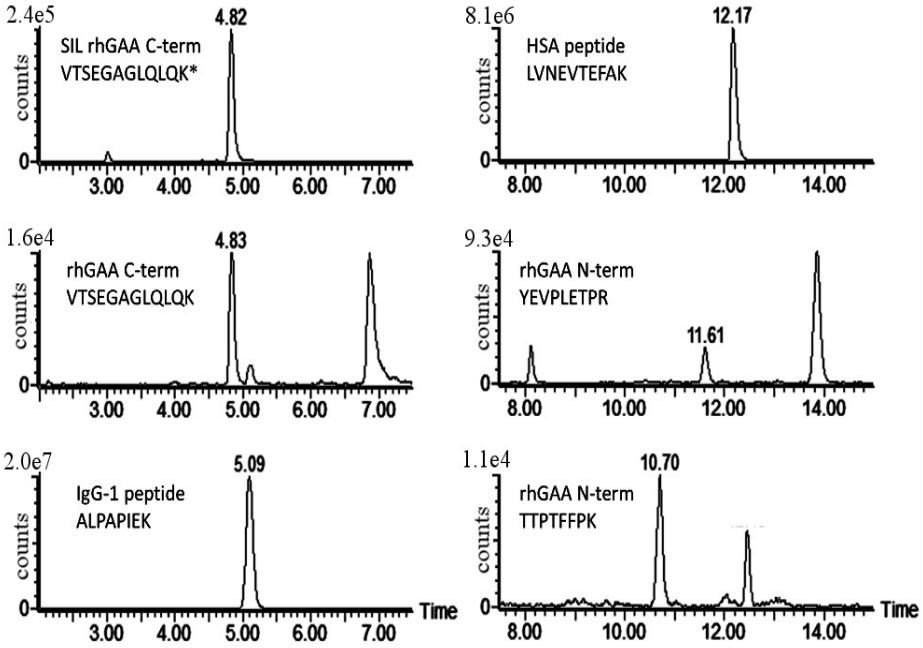

Figure 2. *LC-MS/MS chromatograms of all monitored peptides. In case of multiple peaks the retention times are only shown for the peptide of interest. Taken from a sample which contained 2.50 µg/mL rhGAA*

5.4.4 Protein-G extraction.

To obtain quantitative results for ADA-bound rhGAA, it is essential that the binding capacity of the protein-G material is high enough to bind the entire IgG content, which includes all rhGAA directed ADAs in the applied plasma volume, in a reproducible manner. For our method, the binding capacity (2.2-3mg human IgG per column according to the manufacturer) was evaluated by diluting different volumes of a plasma sample which contained 22.5 µg/mL rhGAA in tenfold diluted ADA plasma (in the range of 6.7 to 80 µL) to 400 µL, the recommended volume to suspend the beads, with load/wash solution and applying this to the protein-G spin columns. The capture efficiency for IgG-1 was determined by comparing the IgG response (signature peptide area) found in the eluate of the protein G beads to that found in directly digested plasma.

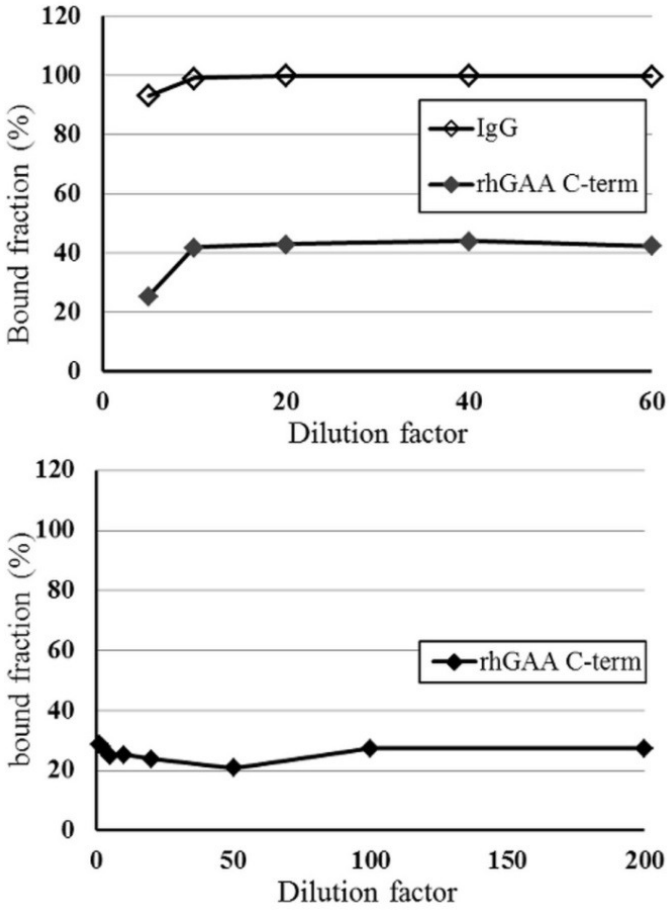

Figure 3. *The effect on the measured bound fraction of dilution with loading buffer (upper pane) and plasma (lower pane).*

In the same experiment, the capture efficiency for ADA-bound rhGAA was assessed by comparing the bound and total rhGAA concentrations. The results from this experiment are shown in the upper pane of Figure 3. The extraction of IgG-1 was essentially complete for dilution factors of at least 10 (corresponding to a plasma volume of up to 40 µL). Application of a larger volume (80 µL) of plasma resulted in a decrease of 10% of captured IgG due to saturation of the protein G beads. For the ADA-rhGAA complex, a similar pattern was observed in which the bound fraction of 42% was constant for dilution factors of 10 and higher (up to 40 µL of plasma). This was reduced to 25% by the application of 80µL of plasma, due to the saturation of the protein-G material.
This indicates that in our method, which uses only 20 µL of plasma, the capture of IgG is complete and also that the equilibrium between rhGAA, its ADAs and the rhGAA-ADA complex is not influenced by the dilution step.

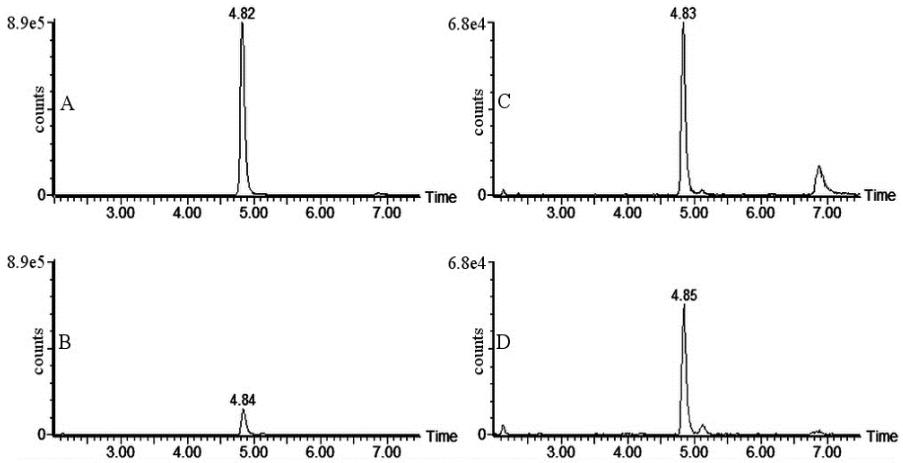

Figure 4. *LC-MS/MS chromatograms of the positive control samples for total (A and C) and ADA-bound (B and D) rhGAA. A and B represent the sample with a low ADA-bound fraction (PC-A), and C and D represent the sample with a high ADA-bound fraction (PC-B).*

In another experiment, it was confirmed that the binding equilibrium of rhGAA and its ADAs is not influenced by dilution of the sample with non-ADA containing plasma, which might be required in case patient samples have an rhGAA concentration above the range of the calibration curve. Total and bound fractions were determined in undiluted plasma with a high ADA-titer spiked with 400 µg/mL rhGAA as well as after up to-200-fold dilution of this sample with blank human plasma, the results are shown in the lower pane of Figure 3.

Although some variability was observed, no trend was seen in the results, which would have been the case if the applied dilutions had an effect on the binding equilibrium between rhGAA and its ADAs. Therefore, ADA-positive plasma can be diluted with blank plasma, e.g. in case of over-curve rhGAA concentrations, without the result for the bound fraction being affected. This also shows that the selected positive control samples (2.5- and 10-fold diluted) are representative for the in vivo situation which may span a wide range of ADA-titers. As an illustration, chromatograms of the positive control samples before and after treatment with protein-G are shown in Figure 4.
For protein quantification methods with LC-MS/MS, a labeled SIL-protein internal standard is usually considered the best option, because it covers the entire procedure. However, for the determination of ADA-bound concentrations, addition of SIL-proteins will alter the ADA-drug equilibrium as the ADAs can also bind to the internal standard. Therefore, in this case, the use of a SIL-peptide internal standard, which does not interfere with the equilibrium, is a more appropriate internal standardization option. However, as the protein-G extraction procedure is not covered by an internal standard, it should be optimized to reduce the variability it introduces. Only then, can a method can be developed with a performance sufficient to be validated according to international guidelines, as was demonstrated in this work. Finally, it should be realized that, just like ELISA-based ADA-titer determinations, the presented approach depends on the presence of antibodies with sufficient affinity towards the drug, because low-affinity antibodies likely release rhGAA during the capture and washing steps of the protein-G extraction. This should not be too much of a concern because low-affinity antibodies are less relevant *in vivo* as they are less likely to impact the therapeutic response.

5.4.5 Protein-G extraction recovery.

Another important quantitative characteristic of the protein-G extraction is the recovery of rhGAA in all fractions collected from the protein-G material. This was determined in ADA-free plasma, in a sample with a low ADA-bound fraction (PC-A) and in a sample with a high ADA-bound fraction (PC-B). The amount of rhGAA was measured before extraction and in the following fractions: the supernatant, the washing solution from two subsequently performed washing steps, and the elution solution. The recovery in each fraction was calculated by comparing the measured peak ratios of the rhGAA peptide and its internal standard from all fractions to those obtained with the total rhGAA method for the same samples (see Figure 5).
The ADA-free sample displays the highest rhGAA recovery (84%) in the supernatant, because all rhGAA is in the free form and no binding to protein G should take place. For this sample, a small part of the analyte (11.8%) is recovered in the first washing step, which must be due to non-specific interactions or

incomplete removal of solvent during the centrifugation step. Only a minor amount of rhGAA (0.1%) was recovered in the second washing step; therefore, the sum of rhGAA recovered in the supernatant and in the first washing step can be considered to represent the free fraction of the drug and only a single washing step was used in the final method for validation and patient sample analysis. As expected, a negligible amount of rhGAA (<2%) was recovered in the elution solvent for the ADA-free sample.

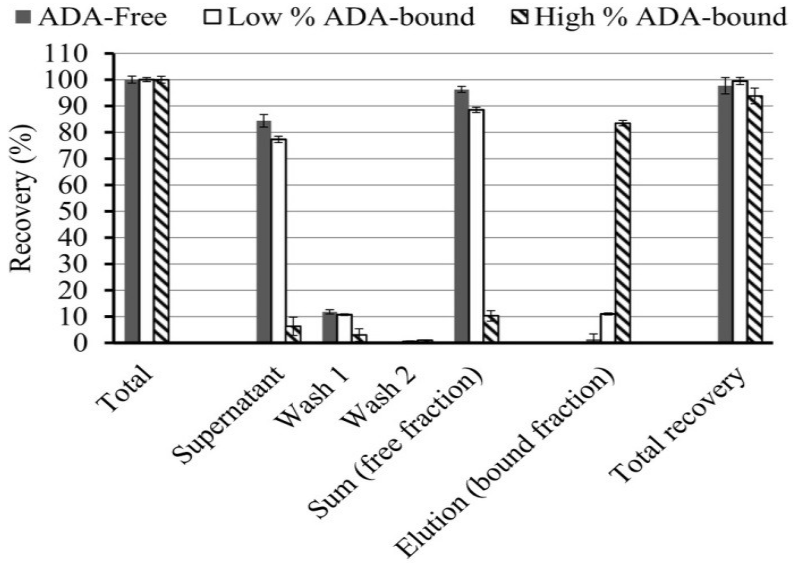

Figure 5. *Recovery of rhGAA in the different fractions of the protein-G extraction for ADA-free samples and samples with a low or a high ADA-bound fraction. For all fractions, recovery is expressed as percentage of the response found in the corresponding result from the total rhGAA analysis, the standard deviation is expressed in error bars.*

The results for the samples with a low and a high ADA bound content are also shown in Figure 5 and indicate the differences in ADA binding (11.0 and 83.5%, respectively). The amount of rhGAA recovered in the first washing step of the low ADA binding sample (~11%) was higher than that found in the high ADA bound sample (~3%); this might be explained by the considerably higher fraction of unbound rhGAA available for binding non-specifically in the sample with a low percentage of ADA-binding. Furthermore, the fact that essentially no analyte was recovered in the second washing step indicates that ADA-bound rhGAA was not released from the ADA-rhGAA complex during the washing procedure, even when a

relatively large amount of ADA-bound rhGAA was present on the beads, as was the case for the sample with a high percentage of ADA-binding.
Importantly, for all samples, the observed total recovery is close to 100%, corresponding to the measured total rhGAA concentration for each sample. This indicates the completeness of elution and underlines the appropriateness of the protein-G extraction for use in a quantitative setting for ADA binding.

5.4.6 Validation.

All obtained results from the validation of the total as well as the ADA-bound rhGAA methods are included in the supplemental tables S1 through S11, a concise summary is shown in Table 2. All results meet the criteria set for small-molecule bioanalytical method validations according to international guidelines[16–18] and thus show that total and ADA-bound concentrations of rhGAA can be determined with high accuracy and precision and that both rhGAA itself and its equilibrium with ADAs is sufficiently stable for a reliable estimation of the ADA binding in patient samples. In addition, sufficient selectivity was observed, because none of the analyzed ADA-containing and ADA-free blank plasma lots showed interferences of more than 20% of the LLOQ response. The results, shown in Table S14, demonstrate that the method is able to accurately quantify rhGAA in protein G extracted plasma against a calibration curve in unextracted plasma, even though these matrices have a different composition.
Next to the bound and total *concentrations*, another important parameter is the *percentage* of rhGAA bound to ADAs, which can be calculated from the measured total and ADA-bound concentrations. Since the analytical variability from both the total and the ADA-bound determinations contributes to the variability of the bound percentage, overall variability may be higher than for the separate concentration measurements. As shown in supplemental tables S8 through S11, the values for the percentage of ADA-bound rhGAA, calculated from the measured bound and total concentrations, were found to be in accordance with the criteria of the validation guidelines, which indicates the suitability of our methodology for the reliable determination of the ADA-bound percentage of rhGAA in human plasma. During the validation of the %-bound rhGAA, a bias of +14.9% was observed in the bench-top stability experiment after 187 hours of storage. Although this is within the acceptable range, it does indicate that the ADA-bound fraction may increase upon prolonged bench-top storage of plasma. To avoid this issue, it is advisable to restrict sample storage at room temperature before analysis.
The efficiency of the protein-G extraction was monitored using the IgG and HSA signature peptides in all samples by comparing the peak area found before and after protein-G extraction, in plasma and eluate, respectively. The extraction recovery of IgG was consistently found to be over 99%, while the amount of HSA found in the

eluate was typically around 5% probably due to non-specific binding to the material (data not shown), which shows the completeness as well as the good selectivity of the IgG extraction.
None of the screened ADA and non-ADA containing blanco plasma lots contained interferences of over 20% of the LLOQ.

Validation item	Maximum bias (%)	Highest CV (%)
Method characteristics		
Accuracy and precision (total rhGAA without ADAs)	-6.8	8.4
Accuracy and precision (total rhGAA with ADAs)	-3.7	4.9
Accuracy and precision (% ADA-bound)	n.a.	11.0
Linearity	-3.8	4.6
Matrix variability	-1.6	5.3
Carry-over (% of response of the LLOQ)	+5.9	n.a.
Matrix effect	n.a.	12.8
Stability of total rhGAA in plasma		
Bench-top (187h)	-5.2	5.4
Freeze-thaw -80°C (3 cycles)	-3.0	5.9
Storage stability -80°C (21 days)	-7.6	2.8
Stability of the % ADA-bound rhGAA in plasma		
Bench-top (187h)	+14.9	4.2
Freeze-thaw -80°C (3 cycles)	+4.5	8.5
Storage stability -80°C (46 days)	+7.1	14.5
Stability of rhGAA in stock solution		
Frozen storage -80°C (546 days)	+3.4	2.6
Bench-top (20 h)	-6.3	8.6
Stability of the signature peptide in extracted plasma digest		
Autosampler 10°C (14 days)	3.3	4.9

Table 2. *Maximal values for bias and CV observed for each of the performed validation experiments*

5.4.7 Patient sample analysis.

The total and the ADA-bound rhGAA levels were determined in several patient samples. The results for two subjects are shown in Figure 6: one subject with a high ADA titer and not responding well to therapy and another subject of comparable age and type of mutation of the gene coding for GAA but without significant ADA titers. As mentioned, the dosing regimen of infusion rates differed slightly between the two subjects although both received the same dose.
High levels of ADA-bound rhGAA were found in the subject with a reduced response to therapy (left pane), compared to the other subject in which almost no ADA-binding was observed (right pane). For all samples, three tryptic peptides of rhGAA were quantified which give information about different parts of the rhGAA molecule. Although the method was only validated for one peptide located near the C-terminal side of the rhGAA molecule (VTSEGAGLQLQK) and no internal standards were used for the other two peptides, the average difference in the measured concentrations between the C-terminal peptide and the peptides TTPTFFPK and YEVPLETPR located near the N-terminus was only -4.4%, and -0.8%, respectively.

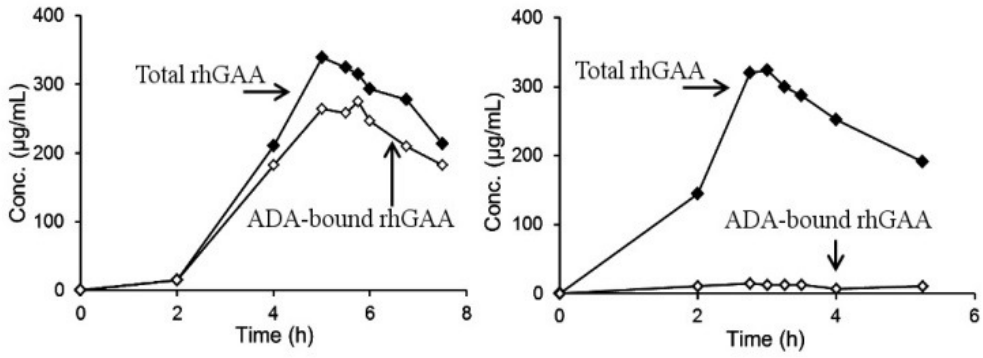

Figure 6. *Pharmacokinetic profiles of total rhGAA (closed symbols) and ADA-bound rhGAA (open symbols) measured in samples from a subject with a reduced response to therapy due to high ADA titers (left pane) as well as in a subject without a significant ADA titer (right pane).*

This indicates that performance of the method for these peptides is adequate to support data interpretation, in the case of unexpected or aberrant results observed for the validated peptide. In the runs in which patient samples were analyzed, one quality control sample for total and one for bound rhGAA was included, the results of which are shown in supplemental table S13 indicating acceptable performance of the assays.

5.5 Conclusions.

An accurate and precise method was developed for the determination of the total and ADA-bound fractions of rhGAA in plasma. The method was optimized to ensure complete trapping of ADA-bound rhGAA by protein-G coated extraction material and to allow dilution of the patient samples with blank plasma and extraction diluent without disturbing the ADA-rhGAA binding equilibrium. The method was validated according to international bioanalytical guidelines and applied to plasma samples from patients undergoing intravenous administration of rhGAA as enzyme replacement therapy.

The good accuracy and precision results obtained for the total rhGAA determinations in samples which contained ADAs indicate that the applied denaturing and digestion protocols effectively removed interferences from ADAs on the measured value for total drug concentrations

The specificity of Protein-G is not limited to human immunoglobulins, which potentially enables the procedure to be used for different species, a feature which for example can be helpful for the assessment of immunogenicity in the pre-clinical phases of biopharmaceutical drug development. The extraction methodology described here is of general value, as protein G is directed to the constant region of all antibodies of the IgG class and will extract all ADA-protein drug complexes from the sample. It can, therefore, be applied for the quantification of the ADA-bound fractions of many other biopharmaceutical drugs, with the exception of IgG-based pharmaceuticals, which bind to protein G in their free form as well. These principles can also be applied to essays where a higher sensitivity is required, which can be achieved by the incorporation of an (immune) extraction following the digestion.

5.6 References

(1) Van den Broek, I.; Niessen, W. M. a; van Dongen, W. D. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* **2013**, *929C*, 161–179.

(2) Ewles, M.; Goodwin, L. *Bioanalysis* **2011**, *3*, 1379–1397.

(3) Bronsema, K. J.; Bischoff, R.; van de Merbel, N. C. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* **2012**, *893-894*, 1–14.

(4) Bronsema, K. J.; Bischoff, R.; Van De Merbel, N. C. *Anal. Chem.* **2013**, *85*, 9528–9535.

(5) Hoofnagle, A. N.; Wener, M. H. *J. Immunol. Methods* **2009**, *347*, 3–11.

(6) Ezan, E.; Bitsch, F. *Bioanalysis* **2009**, *1*, 1375–88.

(7) Wang, S. J.; Wu, S. T.; Gokemeijer, J.; Fura, A.; Krishna, M.; Morin, P.; Chen, G.; Price, K.; Wang-Iverson, D.; Olah, T.; Weiner, R.; Tymiak, A.; Jemal, M. *Anal. Bioanal. Chem.* **2012**, *402*, 1229–39.

(8) Kelley, M.; Ahene, A. B.; Gorovits, B.; Kamerud, J.; King, L. E.; McIntosh, T.; Yang, J. *AAPS J.* **2013**, *15*, 646–58.

(9) Kaliyaperumal, A.; Pennucci, J.; Nagatani, J.; Juan, G.; Swanson, S.; Gupta, S. *J. Pharm. Biomed. Anal.* **2015**, *102*, 176–83.

(10) Neubert, H.; Grace, C.; Rumpel, K.; James, I. *Anal. Chem.* **2008**, *80*, 6907–14.

(11) Heinig, K.; Wirz, T.; Schick, E.; Guenzi, A. *J. Chromatogr. A* **2013**, *1316*, 69–77.

(12) Moreland, R. J.; Jin, X.; Zhang, X. K.; Decker, R. W.; Albee, K. L.; Lee, K. L.; Cauthron, R. D.; Brewer, K.; Edmunds, T.; Canfield, W. M. *J. Biol. Chem.* **2005**, *280*, 6780–91.

(13) De Vries, J. M.; van der Beek, N. A.; Hop, W. C.; Karstens, F. P.; Wokke, J. H.; de Visser, M.; van Engelen, B. G.; Kuks, J. B.; van der Kooi, A. J.; Notermans, N. C.; Faber, C. G.; Verschuuren, J. J.; Kruijshaar, M. E.; Reuser, A. J.; van Doorn, P. a; van der Ploeg, A. T. Effect of enzyme therapy and prognostic factors in 69 adults with Pompe disease: an open-label single-center study. *Orphanet J. Rare Dis.* **2012**, *7*, 73.

(14) Güngör, D.; de Vries, J. M.; Hop, W. C. J.; Reuser, A. J. J.; van Doorn, P. a; van der Ploeg, A. T.; Hagemans, M. L. C. *Orphanet J. Rare Dis.* **2011**, *6*, 34.

(15) Kishnani, P. S.; Corzo, D.; Nicolino, M.; Byrne, B.; Mandel, H.; Hwu, W. L.; Leslie, N.; Levine, J.; Spencer, C.; McDonald, M.; Li, J.; Dumontier, J.; Halberthal, M.; Chien, Y. H.; Hopkin, R.; Vijayaraghavan, S.; Gruskin, D.; Bartholomew, D.; van der Ploeg, A.; Clancy, J. P.; Parini, R.; Morin, G.; Beck, M.; De la Gastine, G. S.; Jokic, M.; Thurberg, B.; Richards, S.; Bali, D.; Davison, M.; Worden, M. A.; Chen, Y. T.; Wraith, J. E. *Neurology* **2007**, *68*, 99–109.

(16) US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Center for Veterinary Medicine. *U.S. Dep. Heal. Hum. Serv. Food Drug Adm. Cent. Drug Eval. Res. Cent. Vet. Med.* **2001**.

(17) European Medicines Agency - Committee for Medicinal Products for

Human Use. Guideline on bioanalytical method validation. **2012**.
(18) Agência Nacional de Vigilância Sanitária - Brazil. Guide for Validation of Analytical and Bioanalytical Methods. *Resolução – RDC No 27.* **2012**.
(19) De Vries, J. M.; van der Beek, N. a M. E.; Kroos, M. a; Ozkan, L.; van Doorn, P. a; Richards, S. M.; Sung, C. C. C.; Brugma, J.-D. C.; Zandbergen, A. a M.; van der Ploeg, A. T.; Reuser, A. J. J. *Mol. Genet. Metab.* **2010**, *101*, 338–45.
(20) Strohalm, M.; Kavan, D.; Novák, P.; Volný, M.; Havlícek, V. *Anal. Chem.* **2010**, *82*, 4648–51.
(21) Altschul, S. F.; Madden, T. L.; Schäffer, a a; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D. J. *Nucleic Acids Res.* **1997**, *25*, 3389–402.
(22) Koomen, J. M.; Haura, E. B.; Bepler, G.; Sutphen, R.; Remily-Wood, E. R.; Benson, K.; Hussein, M.; Hazlehurst, L. a; Yeatman, T. J.; Hildreth, L. T.; Sellers, T. a; Jacobsen, P. B.; Fenstermacher, D. a; Dalton, W. S. *Mol. Cell. Proteomics* **2008**, *7*, 1780–94.
(23) Liu, G.; Zhao, Y.; Angeles, A.; Hamuro, L.; Arnold, M. E.; Shen, J. X. *Anal. Chem.* **2014**, *86*, 8336–43.
(24) Lange, V.; Picotti, P.; Domon, B.; Aebersold, R. *Mol. Syst. Biol.* **2008**, *4*, 222..

5.7 Supplemental materials for chapter 5

A description of the validation experiments for the total glucosidase method.

Acceptance criteria. As per international guidelines, all Bias and CV acceptance criteria were set at 15%, (20% at the LLOQ), except for the stock-stability assessments, for which 10% limits were used.

Linearity. Each validation run contained a calibration curve prepared in human plasma with the following levels: 0.500, 1.00, 2.50, 10.0, 25.0, 100, 250, 400, and 500 µg/mL. The ratio of the measured peak area of the signature peptide over that of the internal standard was used in calculations. Weighted linear regression was applied with 1/xx as weighting factor.

Accuracy and precision. The accuracy and precision of the method were determined by six-fold analysis in three separate runs analyzed on three different days. The validation samples were prepared in plasma at four concentration levels: 0.500, 1.50, 40.0 and 400 µg/mL. Statistical analysis was performed using analysis of variance (ANOVA).

Matrix variability. The influence of the matrix was assessed by preparing samples in eight independent human plasma lots at the LLOQ of 0.500 µg/mL. Additionally, to two of the plasma lots two volume percent of hemolyzed blood cells were added. These were prepared by freezing and thawing the cellular content of whole blood from which the plasma had been removed by centrifugation. The samples were analyzed in one run.

Matrix effect. The effect of the matrix on the obtained responses was assessed by preparing samples in six independent matrix lots at 1.50 and 400 µg/mL. The same concentrations were also prepared in phosphate buffered saline. The plasma samples were analyzed in singlet and the sample in PBS in six-fold in a single run. From each plasma sample a matrix factor was calculated by dividing the peak ratio for plasma by the peak ratio obtained in one of the PBS samples of corresponding concentration. The average matrix effect per concentration level as well as the observed variability between the different matrix lots was used as a measure of the matrix effect at both levels.

As the FDA validation guidelines have been set up for methods which quantify small molecules, the design of the matrix effect experiment does not match protein quantification workflows, which include a digestion step. Spiking extracted and digested plasma with analyte (intact protein), and analyzing these directly is useless as no signature peptides will be present in the sample. Therefore, the effect of the matrix on the entire sample preparation procedure was determined instead. This experiment encompasses the recovery of the precipitation, the digestion efficiency as well as ionization suppression from matrix compounds.

Bench-top stability. Human plasma samples at 1.50 and 400 µg/mL were stored on the bench-top and exposed to normal daylight for 18, 137 and 187 hours, after which the samples were analyzed in a run which contained a freshly spiked and prepared calibration curve.

Freeze-thaw stability. Human plasma samples at 1.50 and 400 µg/mL were stored at -80°C and subjected to three freeze-thaw cycles, in which thawing was performed on the bench-top for a minimum of three hours, followed by frozen storage for at least 12 hours. The samples were analyzed against a freshly spiked and prepared calibration curve.

Frozen stability. Human plasma samples at 1.500 and 400 µg/mL were stored at -80°C for 21 days. The samples were analyzed against a freshly spiked and prepared calibration curve.

Extract stability in the autosampler. Human plasma samples at 1.50 and 400 µg/mL were extracted and analyzed in the first validation run as a part of the accuracy and precision assessment. Subsequently, the extracts were kept in the autosampler at +10°C in the dark for 14 days, after which they were re-analyzed and the concentrations determined against the original calibration curve.

Frozen stability of the stock solution. A 5.0-mg/mL stock solution of glucosidase was prepared from the reference material, and stored at -80°C. 546 days later a second stock solution was prepared freshly. Aliquots from both stock solutions were diluted with plasma to 200 µg/mL. These samples were analyzed in six-fold using the LC-MS/MS methodology for plasma. Stability evaluation was performed by comparing the concentrations found in the samples.

Bench-top stability of the stock solution. An aliquot of the 5.0-mg/mL stock solution of glucosidase was stored on the bench-top, exposed to normal daylight, while another aliquot of the same stock solution was stored at -80°C. After approximately 22 hours both stock solutions were diluted with plasma to 200 µg/mL. These samples were analyzed in six-fold using the LC-MS/MS methodology for plasma. Stability evaluation was performed by comparing the concentrations found in the samples.

Carry-over. For each of the three validation runs which contained accuracy and precision samples, the peak ratios for the three blank plasma samples with internal standard which directly followed the highest calibrator of the calibration curve were compared to the peak ratios of the six validation samples prepared at the LLOQ (0.500 µg/mL) from the same run.

Results of the validation experiments for the total glucosidase method.

Run #	Phase	Concentration (µg/mL) 0.500	1.00	2.50	10.0	25.0	100	250	400	500	Corr. (R^2)	slope	intercept
01	Val	0.499	1.01	2.45	9.87	24.9	98.2	251	417	499	0.9996	0.0098	0.0005
02	Val	0.493	1.05	2.37	9.79	24.5	101	256	417	489	0.9986	0.0390	0.0017
03	Val	0.495	1.01	2.61	9.41	26.0	97.3	255	398	495	0.9987	0.0347	-0.0010
04	Val	0.485	1.04	2.63	9.28	26.9	93.5	262	398	475	0.9981	0.0297	0.0013
05	Val	0.487	1.04	2.58	9.80	26.0	94.6	257	416	460	0.9987	0.0216	0.0024
06	Val	0.502	0.998	2.46	10.1	26.1	92.7	272	403	467	0.9970	0.0249	0.0024
07	Val	0.505	0.975	2.52	10.3	26.5	97.3	254	410	453	0.9988	0.0250	0.0024
08	Val	0.505	0.975	2.52	10.3	26.5	97.3	254	410	453	0.9988	0.0250	0.0024
09	Val	0.526	0.908	2.41	9.91	26.0	101	250	408	507	0.9989	0.0282	0.0002
Average		0.499	1.00	2.50	9.81	25.9	96.9	257	408	481	0.9985	0.0266	0.0012
Bias (%)		-0.2	+0.4	+0.2	-1.9	+3.5	-3.1	+2.9	+2.1	-3.8	n.a.	n.a.	n.a.
CV (%)		2.6	4.6	3.8	3.4	3.1	3.2	2.7	2.0	4.1	n.a.	n.a.	n.a.

Table S1. *Individual calibration curve results from the validation (Val). The calculation of average, bias and coefficient of variation (CV) values was performed before rounding. All values were taken from the data-processing software Masslynx 4.1.*

Run	Concentration (µg/mL)						Average	Bias	CV
	Nominal concentration 0.500						µg/mL	(%)	(%)
01	0.453	0.554	0.548	0.463	0.527	0.513	0.510	+1.9	8.4
02	0.491	0.519	0.462	0.529	0.538	0.545	0.514	+2.8	6.2
03	0.462	0.516	0.481	0.536	0.497	0.558	0.508	+1.7	7.0
						Total	0.511	+2.1	6.3
	Nominal concentration 1.50						ng/ mL	(%)	(%)
01	1.59	1.60	1.61	1.59	1.59	1.50	1.58	+6.0	2.6
02	1.54	1.62	1.55	1.62	1.53	1.58	1.58	+5.0	2.6
03	1.49	1.57	1.50	1.33	1.49	1.56	1.49	-0.6	5.9
						Total	1.55	+3.2	4.6
	Nominal concentration 40.0						ng/ mL	(%)	(%)
01	38.0	35.1	38.5	38.3	38.5	39.5	38.0	-5.0	3.9
02	39.2	40.7	41.8	40.9	41.9	41.1	40.9	+2.3	2.4
03	37.8	38.7	37.7	38.4	37.6	37.8	38.0	-5.0	1.2
						Total	39.0	-2.6	4.4
	Nominal concentration 400						ng/ mL	(%)	(%)
01	379	379	386	391	399	393	388	-3.0	2.1
02	395	402	408	405	409	397	402	+0.6	1.4
03	364	375	369	378	372	379	373	-6.8	1.6
						Total	388	-3.1	3.6

Table S2. *Individual accuracy and precision results from the total rhGAA plasma validation. The calculation of average, bias and coefficient of variation (CV) values was performed before rounding the values.*

Plasma Lot												
1*	2*	3	4	5	6	7	8	9	10	Average	Bias	CV
Nominal concentration 10.0 (µg/mL)										µg/ mL	(%)	(%)
0.472	0.460	0.507	0.492	0.499	0.546	0.467	0.473	0.514	0.491	0.492	-1.6	5.3

*: 2% of hemolyzed blood cells added to the sample before spiking.

Table S3. *Matrix variability results from the total rhGAA plasma validation. The calculation of average, bias and coefficient of variation (CV) values was performed before rounding the values.*

Time (h)	Concentration (µg/mL) nominal 1.50			Average µg/ mL	Bias (%)	CV (%)
Bench-top stability						
18	1.53	1.43	1.59	1.52	+1.1	5.4
137	1.42	1.44	1.55	1.47	-2.0	4.9
187	1.61	1.55	1.51	1.56	+3.8	3.1
Freeze-thaw stability (3 cycles)						
-	1.52	1.61	1.48	1.54	+2.4	4.5
Frozen plasma sample stability (14 days at -80°C)						
-	1.49	1.57	1.50	1.5	+1.5	2.8
Extracted plasma digest stored in the autosampler (14 days)						
-	1.51	1.63	1.50	1.5	+3.1	4.9

Time (h)	Concentration (µg/mL) nominal 400			Average µg/ mL	Bias (%)	CV (%)
Bench-top stability						
18	364	392	403	386	-3.4	5.3
137	357	391	390	379	-5.2	5.1
187	390	389	379	386	-3.5	1.6
Freeze-thaw stability (3 cycles)						
-	407	363	393	388	-3.0	5.9
Frozen plasma sample stability (14 days at -80°C)						
-	364	375	369	370	-7.6	1.5
Extracted plasma digest stored in the autosampler (14 days)						
-	412	417	410	413	+3.3	0.8

Table S4. *Results from the stability experiments conducted in total rhGAA plasma validation. The calculation of average, bias and coefficient of variation (CV) values was performed before rounding the values.*

Item	Ratio over internal standard						Average	Bias	CV
Stock stability, fresh versus stored at -80°C for 546 days							(ratio)	(%)	(%)
Fresh	0.614	0.621	0.642	0.650	0.643	0.645	0.636	reference	2.2
Stored	0.642	0.670	0.673	0.655	0.674	0.632	0.658	+3.4	2.6
Stock stability, stored on BT versus stored at -80°C for 20 hours						(ratio)	(%)	(%)	
Fresh	0.843	0.704	0.682	0.699	0.690	0.690	0.718	reference	8.6
Stored	0.677	0.637	0.682	0.711	0.643	0.687	0.673	-6.3	4.2
Stock reproducibility, two separate vials dissolved and compared						(ratio)	(%)	(%)	
Stock 1	0.611	0.607	0.629	0.648	0.610	0.629	0.622	reference	2.6
Stock 2	0.614	0.621	0.642	0.650	0.643	0.645	0.636	+2.2	2.3

Table S5. *Results of the stock stability and reproducibility experiments conducted in the human plasma validation. The calculation of average, bias and coefficient of variation (CV) values was performed before rounding the values.*

Run	Ratios of the LLOQ's						Average.	Ratios of the zero samples			Average	% carry-over
01	0.005	0.006	0.006	0.005	0.006	0.006	0.006	0.001	0	0	0.0003	5.9
02	0.021	0.022	0.02	0.022	0.023	0.023	0.022	0.001	0.001	0	0.0007	3.1
03	0.015	0.017	0.016	0.018	0.016	0.018	0.017	0	0	0.001	0.0003	2.0

Table S6. *Results of the carry-over experiments conducted in the human plasma validation. The calculation of the average, and the percentage carry over was performed before rounding the values.*

Nominal concentration 1.50 µg/mL

Lot (#)	1	2	3	4	5	6	Average	CV (%)
plasma (ratio)	0.06	0.06	0.06	0.059	0.058	0.064	-	-
buffer (ratio)	0.034	0.035	0.036	0.043	0.038	0.032	-	-
Matrix factor	1.76	1.71	1.67	1.37	1.53	2.00	1.67	12.8

Nominal concentration 400 µg/mL

lot (#)	1	2	3	4	5	6	Average	CV (%)
plasma (ratio)	14.7	14.3	15.3	15.2	14.5	14.5	-	-
buffer (ratio)	2.44	2.31	2.25	1.98	1.93	1.80	-	-
Matrix factor	6.01	6.19	6.83	7.70	7.51	8.06	7.05	11.9

Table S7. *Results of the matrix effect experiments conducted in the total GAA plasma validation. The calculation of average, bias and coefficient of variation (CV) values was performed before rounding the values.*

Description of the validation experiments for the ADA-bound glucosidase method.

Accuracy and precision. The accuracy and precision of the method were determined by six-fold analysis in three separate runs analyzed on three different days of the PC-A and PC-B samples. Statistical analysis was performed using analysis of variance (ANOVA).
The average results from these experiments were used as nominal values in the experiments to determine the stability of the complex.

Bench-top stability. Aliquots of PC-A and PC-B were stored on the bench-top and exposed to normal daylight for 18, 137 and 187 hours, after which the samples were analyzed in a run which contained a freshly spiked and prepared calibration curve and QC samples.

Freeze-thaw stability. Aliquots of PC-A and PC-B were stored at -80°C and subjected to three freeze-thaw cycles, in which thawing was performed on the bench-top for a minimum of three hours, followed by frozen storage for at least 12 hours. The samples were analyzed the samples were analyzed in a run which a contained freshly spiked and prepared calibration curve and QC samples.

Frozen stability. Aliquots of PC-A and PC-B were stored at -80°C for 46 days. The samples were analyzed the samples were analyzed in a run which a contained freshly spiked and prepared calibration curve and QC samples.

Run	Response					Average		Bias	CV
	Total concentration (nominally 125 µg/mL)						µg/mL	(%)	(%)
01	122	119	119	120	120	122	120	-3.7	1.2
02	119	124	130	130	128	128	126	+1.1	3.4
03	119	122	126	121	128	129	124	-0.7	3.1
						Total	124	-1.1	3.3
	ADA-bound concentration						µg/mL		(%)
01	14.0	13.7	12.2	14.3	13.4	12.8	13.4	-	6.0
02	13.9	15.0	14.9	15.3	13.8	15.1	14.7	-	4.4
03	14.5	12.8	14.0	13.6	12.8	14.7	13.7	-	6.1
						Total	13.9	-	6.5
	Calculated percentage ADA bound						%		(%)
01	11.5	11.5	10.2	11.9	11.2	10.4	11.1	-	6.0
02	11.7	12.1	11.5	11.8	10.8	11.8	11.6	-	3.9
03	14.5	12.8	14.0	13.6	12.8	14.8	13.8	-	6.1
						Total	12.2	-	11.0

Table S8. *Individual accuracy and precision results from the PC-A sample. The fraction ADA-bound rhGAA was calculated by comparing the measured total and ADA-bound concentrations. The calculation of average, bias and coefficient of variation (CV) values was performed before rounding the values.*

Run	Response						Average	Bias	CV
	Total concentration (nominally 9.23 µg/mL)						µg/mL	(%)	(%)
01	9.20	8.92	9.22	9.00	9.17	*	9.10	-1.6	1.5
02	9.45	8.58	9.18	9.75	9.54	9.83	9.39	+1.5	4.9
03	9.35	9.54	9.34	9.34	9.34	9.83	9.46	+2.2	2.1
						Total	9.33	+0.9	3.5
	ADA-bound concentration						µg/mL		(%)
01	7.71	7.59	7.36	7.80	7.27	*	7.55	-	3.0
02	7.41	7.46	7.44	7.92	8.04	7.10	7.56	-	4.7
03	9.13	7.44	8.13	7.47	8.05	8.10	8.05	-	7.6
						Total	7.73	-	6.2
	Calculated percentage ADA bound						%		(%)
01	83.8	85.1	79.9	86.7	79.3	*	82.9	-	3.9
02	78.4	86.9	81.0	81.3	84.3	72.3	80.7	-	6.3
03	97.6	78.0	87.0	79.9	86.2	82.3	85.2	-	8.2
						Total	82.9	-	6.6

* sample preparation error.

Table S9. *Individual accuracy and precision results from the PC-B sample. The fraction ADA-bound rhGAA was calculated by comparing the measured total and ADA-bound concentrations. The calculation of average, bias and coefficient of variation (CV) values was performed before rounding the values.*

Concentration (µg/mL)				Average	Bias	CV
PC-A (Nominally 125)				µg/mL	(%)	(%)
Bench-top stability (20h)						
Total	133	131	130	131	+6.1	1.0
ADA-bound	16.6	17.5	16.5	16.9	+14.9	3.6
ADA-bound (%)	11.6	12.6	12.3	12.2	+0.3	4.2
Freeze-thaw stability (3 cycles)						
Total	126	116	122	121	-2.0	4.0
ADA-bound	15.9	15.0	14.8	15.2	+9.3	3.6
ADA-bound (%)	12.6	12.9	12.1	12.6	+3.3	3.0

Concentration (µg/mL)				Average	Bias	CV
PC-B (nominally 9.23)				µg/mL	(%)	(%)
Bench-top stability (20h)						
Total	9.99	9.81	9.61	9.81	+5.1	1.9
ADA-bound	8.03	8.24	8.05	8.11	+4.9	1.4
ADA-bound (%)	80.4	84.0	83.7	82.7	-0.3	2.4
Freeze-thaw stability (3 cycles)						
Total	9.27	9.14	9.58	9.3	0.0	2.4
ADA-bound	8.60	8.11	7.52	8.1	+4.5	6.7
ADA-bound (%)	92.7	88.7	78.5	86.6	+4.5	8.5

Table S10. *Results from the bench-top and freeze-thaw stability experiments conducted in the ADA-bound rhGAA human plasma validation. The calculation of average, bias and coefficient of variation (CV) values was performed before rounding the values. All bias values are calculated from the average measured values from the accuracy and precision experiments which were conducted with the same samples.*

Concentration (µg/mL)				Average	Bias	CV
PC-A (Nominally 125)				µg/mL	(%)	(%)
Frozen plasma sample stability (46 days)						
Total	125	124	115	121	-2.9	4.9
ADA-bound	16.0	16.3	15.1	15.8	+13.5	3.9
ADA-bound (%)	12.8	13.1	13.2	13.0	+7.1	1.7

Concentration (µg/mL)				Average	Bias	CV
PC-B (nominally 9.23)				µg/mL	(%)	(%)
Frozen plasma sample stability (46 days)						
Total	8.86	9.06	9.06	9.08	-2.7	2.5
ADA-bound	8.74	6.81	7.29	7.61	-1.5	13.2
ADA-bound (%)	98.6	71.1	80.4	84.8	2.2	14.5

Table S11. *Results from the frozen storage stability experiments conducted in the ADA-bound rhGAA human plasma validation. The calculation of average, bias and coefficient of variation (CV) values was performed before rounding the values. All bias values are calculated from the average measured values from the accuracy and precision experiments which were conducted with the same samples.*

Results of the total rhGAA Quality control samples in the ADA-bound rhGAA validation and bioanalysis

1.50 µg/mL		Average µg/mL	Bias %	CV %
1.44	1.41	1.42	-5.1	1.8
1.49	1.59	1.54	+2.6	4.2
1.64	1.46	1.55	+3.1	8.2
1.66	1.54	1.60	+6.8	5.5
1.66	1.51	1.58	+5.6	6.4
1.44	1.47	1.45	+0.9	1.4
1.50	1.67	1.58	+9.7	7.6
1.61	[1.74]	1.68	[+16.3]	5.4
1.44	[1.74]	1.59	+10.4	13.2
1.56	1.66	1.61	-2.7	4.4
	Average	1.56	+6.8	4.1

40.0 ug/mL		Average µg/mL	Bias %	CV %
39.2	38.2	38.7	-3.3	1.9
44.3	44.0	44.2	+10.4	0.5
42.3	41.9	42.1	+5.1	0.7
40.4	43.8	42.1	+5.3	5.7
38.7	40.4	39.5	-1.1	3.2
42.9	41.2	42.1	+5.1	2.8
40.0	43.1	41.6	+4.0	5.3
41.0	43.1	42.0	+5.1	3.5
38.7	43.0	40.9	+2.2	7.4
41.0	41.8	41.4	+3.5	1.4
	Average	41.5	+4.5	3.6

40.0 ug/mL		Average µg/mL	Bias %	CV %
371	373	372	-6.9	0.4
435	421	428	+7.1	2.3
419	416	417	+4.3	0.5
418	446	431	+8.0	4.5
422	397	410	+2.5	4.3
424	421	422	+5.6	0.5
395	362	379	-5.3	6.2
416	421	419	+4.7	0.8
404	413	409	+2.2	1.6
389	417	403	+0.7	4.9
	Average	409	+5.3	2.3

***Table S12**. Results of the total rhGAA quality control samples which were measured in each run which did not contain accuracy and precision samples of the total rhGAA method. The QC samples were analyzed in two sets, each of which contained the three QC levels in ascending concentration. The calculation of average, bias and coefficient of variation (CV) values was performed before rounding the values. The bracketed values are out of the set limits, no runs were rejected.*

PC-A (Nominally 125)		PC-B (Nominally 9.23)	
Total (µg/mL)	116	Total (µg/mL)	8.36
ADA-bound (µg/mL)	12.5	ADA-bound (µg/mL)	7.67
ADA-bound (%)	10.8	ADA-bound (%)	91.7
Bias (%)	-11.2	Bias (%)	+10.6

Table S13. *Results of the free rhGAA quality control samples which were measured the bioanalytical run. The calculation of the bias values was performed before rounding the values. The bias was calculated from the average measured values from the accuracy and precision experiments which were conducted with the same samples.*

Matrix	Concentration (µg/mL) nominal 1.50			Average	Bias (%)	CV (%)	Concentration (µg/mL) nominal 400			Average	Bias (%)	CV (%)
Eluate	1.62	1.63	1.72	1.66	+10.4	3.3	396	418	404	406	1.5	2.7

Table S14. *Performance of the method in the eluate from the protein-G extraction, concentrations calculated using a calibration curve in untreated plasma. The calculation of the bias values was performed before rounding the values. The bias was calculated from the nominal value.*

Good manners will open doors that the best education cannot

Clarence Thomas